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miR-296 and its target Scrib in breast cancer

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ABSTRACT

microRNAs (miRNAs) are small non-coding RNAs which negatively regulate the expression of several mRNAs at post-transcriptional level. miRNAs are involved in multiple physiological processes (development, differentiation and cell growth, apoptosis, etc.) and their aberrant expression has been reported in several types of diseases, including tumors.

miR-296 plays important roles in different cell types and cellular pathways, regulating angiogenesis, stem cell differentiation, cell tumorigenic potential and motility. These latter two functions are potentially mediated by inhibition of one of its target, Scrib, a cytoplasmic protein involved in apico-basal cell polarity maintenance and in cell motility. In tumors, the role of miR-296 and of its target Scrib still remains debated, and scarce data are available in breast carcinomas.

Given the role of miR-296 in control of physiological processes normally deregulated in tumors, we aimed to evaluate the expression levels of miR-296 by Real Time PCR and of its target Scrib by immunohistochemistry, in a large series of breast cancers to identify any correlations with clinical and pathological parameters, useful for prognostic and/or predictive purposes, and to clarify the role of this miRNA and its target in mammary tumorigenesis.

Finally, to evaluate if modification of miR-296 levels could have effects on neoplastic growth, we injected breast cancer cells (MDA-MB 231), implanted in a mouse model, with a precursor of miR-296 or a control molecule monitoring tumor growth in time.

miR-296 levels were consistently reduced in human breast cancer tissues compared with non-neoplastic mammary parenchyma. In the subgroup of patients with distant metastases, a significant correlation between reduced miR-296 levels and earlier spread of cancer was detected.

Regarding miR-296 target, breast cancers displayed higher levels of Scrib compared to normal parenchyma, and with a greater intensity.

Direct injection of pre-miR-296 into tumoral masses of a xenograft model significantly decreased tumor growth.

Our data confirm the involvement of miR-296 in breast cancer tumorigenesis, in particular, a significant reduction is observed in infiltrating carcinomas compared to normal controls and experimentally induced restoration of its levels in xenograft assays resulted effective in slowing tumor growth. Furthermore, our observation in the group of patients with distant metastases, showing a significant correlation between reduced miR-296 levels and the earlier onset of distant metastases, highlights the role of miR-296 as a regulator of cell migration and invasion.

With this background, miR-296 could represent a new therapeutic approach and provide a scientific rationale for the design of new drugs with molecular target, appropriate to restore the levels of miRNAs and induce anti-neoplastic effects.

SOMMARIO

I microRNA (miRNA) sono piccoli RNA non codificanti che regolano negativamente l'espressione di numerosi mRNA a livello post-trascrizionale. I microRNA sono coinvolti in svariati processi fisiologici (sviluppo, crescita e differenziazione cellulare, apoptosi, etc) e la loro espressione aberrante è stata riportata in vari tipi di malattie, compresi i tumori.
miR-296 svolge un ruolo importante in diversi tipi di cellule, controllando l'angiogenesi, il differenziamento delle cellule staminali, il potenziale oncogenico e la motilità. Queste ultime due funzioni sono potenzialmente mediate dall' inibizione di un suo target, Scrib, una proteina citoplasmatica coinvolta nel mantenimento della polarità apico-basale e nella motilità delle cellule. Nei tumori il ruolo di miR-296 e del suo target Scrib rimane ancora dibattuto e pochi dati sono disponibili riguardo i carcinomi della mammella.
Data la partecipazione di miR-296 nella regolazione di processi fisiologici di solito alterati nei tumori, abbiamo deciso di valutare i livelli di espressione di miR-296 mediante Real Time PCR e del suo target Scrib mediante immunoistochimica in un'ampia serie di tumori mammari per individuare eventuali correlazioni con i parametri clinici e patologici, utili a fini prognostici e / o predittivi, e per chiarire il ruolo di questo miRNA e del suo target nella tumorigenesi mammaria.
Infine, per valutare se modificando i livelli di miR-296 si potevano avere effetti sulla crescita neoplastica, abbiamo iniettato cellule tumorali mammarie (MDA-MB 231), impiantate in un modello di xenotrapianto, con un precursore di miR-296 o una molecola di controllo e monitorato nel tempo la crescita tumorale.
I nostri dati mostrano che i livelli di miR-296 sono costantemente ridotti nei carcinomi della mammella rispetto al parenchima mammario sano. Inoltre, nel sottogruppo di pazienti con metastasi a distanza, è stata osservata una correlazione significativa tra la riduzione dei livelli di miR-296 e la disseminazione precoce del tumore.
Per quanto riguarda il target di miR-296, Scrib è risultato espresso in un numero maggiore di casi nei carcinomi mammari rispetto al parenchima normale e con un'intensità maggiore.
L'iniezione diretta di miR-296 in cellule di carcinoma mammario umano impiantate in un modello di xenotrapianto ha inoltre ridotto la crescita del tumore rispetto ai controlli.
I nostri dati confermano il coinvolgimento di miR-296 nella tumorigenesi mammaria, in particolare, una riduzione significativa dei suoi livelli è stata osservata nei carcinomi rispetto ai controlli normali e la sua forzata riespressione nel modello di xenotrapianto è risultata efficace nel rallentare la crescita tumorale. Inoltre, l'osservazione che nel gruppo dei pazienti con metastasi a distanza esiste una correlazione significativa tra riduzione dei livelli di miR-296 e lo sviluppo precoce delle metastasi mette in evidenza il ruolo di miR-296 come regolatore della migrazione e dell'invasività cellulare.
Con queste premesse, miR-296 potrebbe rappresentare un nuovo target per la progettazione di farmaci mirati, atti a ripristinare i livelli di miRNA e ad indurre effetti anti-neoplastici.

SYMBOL LIST

aPKC: atypical protein kinase C
ASCO: American Society of Clinical Oncology
BIC: B cell integration cluster
BCL-2: B-cell chronic lymphocytic leukemia/lymphoma 2
BRMS1: breast cancer metastasis suppressor 1
CAP: College of American Pathologists
CDK4: cyclin-dependent kinase 4
CpG: cytosine nucleotide occurs next to a guanine nucleotide in the linear sequence of bases, is shorthand for "—C—phosphate—G—"
DFS: disease-free survival
DICER: endoribonuclease Dicer
DGCR-8: DiGeorge syndrome critical region 8
Dlg: Discs large protein
DROSHA: ribonuclease 3
EGF: epidermal growth factor
EMT: epithelial mesenchymal transition
E6AP: E6 associated protein
ER: estrogen receptor
FMRP: fragile X mental retardation protein
GNAS complex locus: guanine nucleotide binding protein, alpha stimulating activity polypeptide 1
GW182: glycine-tryptophan repeat-containing protein of 182 kDa
HCV: hepatitis C virus
HER2: human epidermal growth factor receptor-2
HGS: hepatocyte growth factor-regulated tyrosine kinase substrate
HPV: human papilloma virus
IHC: immunohistochemistry
IFNα and β: interferon α and β
LAP: LRR and PDZ domains
Let-7: lethal-7 gene
Lgl: Lethal giant larvae protein
LRR: Leucine–Rich Repeat
MAPK: mitogen activated protein kinase
MET: hepatocyte growth factor receptor gene
MID domain: middle domain
miR: microRNA
miRISC: RNA-induced silencing complex carrying miR
MOV10: Moloney leukemia virus 10 protein
mRNA: messenger ribonucleic acid
MYC: v-myc myelocytomatosis viral oncogene homolog
Nanog: homeobox transcription factor Nanog
NOTCH3: Neurogenic locus notch homolog 3
Oct4: octamer-binding transcription factor 4
OS: overall survival
PACT: protein activator of the interferon induced protein kinase
PALS: protein associated with Lin7
Par3: Partitioning defective 3 protein

Par6: Partitioning defective 6 protein
PATJ: tight-junction associated PDZ protein
PAZ domain: Piwi/Argonaute/Zwille domain
PDCD4 : programmed cell death 4 gene
PDGFR-β: platelet derived growth factor receptor β
PDZ: post synaptic density protein 95 (PSD95), Drosophila Disc large tumor suppressor 1 (Dlg-1), zonula occludens -1 protein (zo1)
PIWI domain: P-element induced wimpy testis domain
pri-miRNA: primary micro-RNA
pre-miR: micro-RNA precursor
PR: progesterone receptor
RAS: Rat sarcoma gene
Real Time PCR: quantitative polymerase chain reaction
RIP-Tag transgenic mice: rat insulin II promoter-simian virus 40 large T antigen
RISC: RNA-induced silencing complex
RLC: RISC loading complex
RNA: ribonucleic acid
RNU48: small nucleolar RNA, C/D box 48
SMAD: SMA (small body size) and MAD (mothers against decapentaplegic)
SMN complex: survival of motor neurons complex
Sox2: SRY (sex determining region Y)-box 2
Sox4: SRY (sex determining region Y)-box 4
3'UTR: the 3' untranslated region
TGF β: transforming growth factor β
TMA: tissue micro-array
TNM: classification of malignant tumours
TNRC6A, 6B, 6C: trinucleotide repeat containing 6A,6B,6C proteins
TRBP: trans-activator RNA-binding protein
Tudor-SN: Tudor staphylococcal nuclease-domain-containing protein
TWIST: twist homolog 1 gene
VEGFR2: vascular endothelial receptor-2
ZEB1: zinc finger E-box binding homeobox 1
ZEB2: zinc finger E-box binding homeobox 2

1. INTRODUCTION

1.1 Breast cancer

Breast cancer is the most frequent malignancy in women, and still remains the leading cause of cancer death in women despite many improvements in early diagnosis and availability of targeted treatment [1]

In recent years, the advent of high-throughput platforms for analysis of gene expression, such as microarrays, has changed the idea that breast cancer is a single disease with varying histopathological features and clinical behaviour. Breast cancer is now perceived as a heterogeneous disease, encompassing about 15 different types of carcinomas, that affect the same organ site and originate from the same anatomical structure (i.e. the terminal duct lobular unit), but have different risk factors, clinical presentation, histopathological features, outcome, and response to systemic therapies [2-6].

At least five molecular categories (luminal A/B, HER-2-like, normal breast-like, and basal-like) have been identified in breast carcinomas, each with prognostic significance [2].

The classification of breast cancers into subgroups on the basis of gene expression patterns in tumour tissue is often limited, due to the expense and technical difficulty encountered when carrying out high-throughput gene-expression profiling using paraffin-embedded material.

Consequently, more easy to use, immunohistochemical (IHC) markers to classify tumors into subtypes that are surrogates for those based on gene-expression profiling have been implemented into international guidelines [7,8].

Currently, estrogen receptor (ER), progesterone receptor (PR) status and human epidermal growth factor receptor-2 (Her-2) status are routinely used as predictive markers to select specific adjuvant therapies. Prognostic markers may also be used to target adjuvant chemotherapy, such as tumour size, presence of lymph-node metastasis, and histological grade. These factors are combined in the form of algorithms for treatment decision making [8].

Besides clinical and pathological parameters, identification of molecular markers prognostically and therapeutically relevant is becoming even more important to offer a personalized disease management and treatment options.

Signalling pathways involved in breast carcinogenesis have been extensively explored.

In recent years, it has been shown that small non-coding RNAs (microRNA) are aberrantly expressed or mutated in cancer, suggesting that they may play a role as a novel class of oncogenes or tumor suppressor genes.

Application of these markers in the clinical setting could have the potential to improve the targeting therapy to those most likely to benefit, for implementation of individualised therapy.

1.2 MicroRNAs (miRNAs)

MicroRNAs (miRNAs) are small non-coding RNAs which negatively regulate the expression of several mRNAs at post-transcriptional level by inhibiting their translation or stimulating degradation [9].
This type of regulation was first described in *Caenorhabditis elegans* in 1993 [10,11] and it has since been described in many other organisms. Today, more than 1,400 miRNAs have been described in humans (miRBase - http://www.mirbase.org/cgi-bin/browse.pl), constituting 1 to 3% of the genes in the human genome [12]. It has been estimated that miRNAs regulate 30 to 60% of protein-coding genes [9,13].

1.2.1 miRNAs biogenesis

miRNAs are located in different regions of the genome; 70% are intergenic, but they are also found within exonic or intronic regions in either sense or antisense orientation [14]. miRNAs localized within introns of protein-encoding or -non-encoding genes have been denominated 'mirtrons' [15]. Forty percent of all miRNAs are organized in clusters, within 0.1 and 50 kb from each other, and usually regulate a common pathway [16,17].
miRNAs are mainly transcribed by RNA polymerase II from their own promoter or from promoter of the host gene in which they reside, to generate long primary transcripts (pri-miRNAs), which can have one or several secondary structures with 60- to 80-nt loops [18]. Clustered miRNAs might be transcribed from a single transcription unit as polycistronic primary-miRNA.
Pri-miRNAs are cleaved in the nucleus by the microprocessor complex, containing Drosha, an RNase III endoribonuclease, and it's partner DiGeorge syndrome critical region 8 (DGRC8), a double stranded RNA-binding protein, to form precursor miRNAs (pre-miRNAs) [19].
Pre-miRNAs, hairpin structures of approximately 70 nt with a 2-nt 3' overhang [20], are then exported from the nucleus to the cytoplasm by exportin-5 and its co-factor Ran-GTP [21] and are further incorporated into the RISC Loading Complex (RLC) where are processed by the type III ribonuclease Dicer into a ~21-nucleotides-long miRNA/miRNA* duplex [22,23].
Transitory miRNA duplex includes the mature miRNA guide (miRNA), generally selected according to thermodynamic properties, and the complementary passenger strand (miRNA*), usually subjected to degradation, or incorporated into specific exosomes and extruded from the cell to constitute the circulating pool of miRNAs [24]. However, whereas the complementary passenger strand was initially thought to be the strand subjected to degradation, instead more recent evidences suggest that it does not simply represent a non-functional product of miRNA biogenesis, but it can be selected as a functional strand and play significant biological roles [25].
Once mature, miRNAs become an integral part of the effector RNA-induced silencing complex (RISC), a multiprotein complex which keeps only the strand that is less stable at its 5' end and subsequently initiates the post-transcriptional gene silencing.
The miRNA-carrying RISC (miRISC) includes Dicer, the RNA-binding Argonaute proteins, and the adaptor protein trans-activator RNA-binding protein (TRBP), a double-stranded RNA-binding protein [26].

Main RISC components are Argonaute proteins (Ago1-4), containing three evolutionarily conserved domains, PAZ, MID, and PIWI, which interact with the 3' and 5' ends of the miRNA respectively, and mediate binding of miRNAs to RISC [27-29].
Ago-2 can act specifically to cleave target RNA. The specific role of Ago1, 3, and 4 in miRNA function remains elusive [30].
GW182 proteins (glycine-tryptophan repeat-containing protein of 182 kDa) are another group of factors crucial for the miRNA-induced repression. They interact directly with and act downstream of Argonaute proteins as silencing effectors. There are three mammalian GW182 proteins known as TNRC6A, -B, and –C [31].
miRISC interacts with several additional proteins that may function as regulatory factors that modulate miRNA function. Additional recruited proteins to RISC complex are PACT (protein activator of the interferon induced protein kinase), a paralogue of TRBP required for efficient miRNA accumulation in vivo and positive regulator of RNA silencing; two RNA helicase Gemin3, and Gemin4, components of the SMN complex (survival of motor neurons complex); FMRP (fragile X mental retardation protein) a RNA binding protein known to act as modulator of translation; a nuclease Tudor-SN (Tudor staphylococcal nuclease-domain-containing protein), and MOV10 (Moloney leukemia virus 10 protein) required for RNA silencing [32-35].
Generally, one strand of the intermediate miRNA duplex is loaded into RISC [36,37]. However, some miRNA-encoding loci can incorporate both strands into RISC.
Alternatively, miRNAs may be processed from within introns of protein-encoding or -non-encoding genes to generate pre-miRNAs directly [38].
Canonical mirtrons are processed co-transcriptionally before splicing, and the splicing commitment complex is thought to bind the introns while Drosha cleaves the miRNA hairpin. At this point, the precursor miRNA enters the classical miRNA pathway, whereas the rest of the transcript undergoes precursor mRNA splicing and produces mature protein-coding mRNA. In non-canonical pathway, mirtrons are produced from spliced introns as debranched introns that mimic the structural features of precursor miRNAs and enter to miRNA-processing pathway without Drosha-mediated cleavage [15,39-41].
miRNAs biogenesis is illustrated in Figure 1.

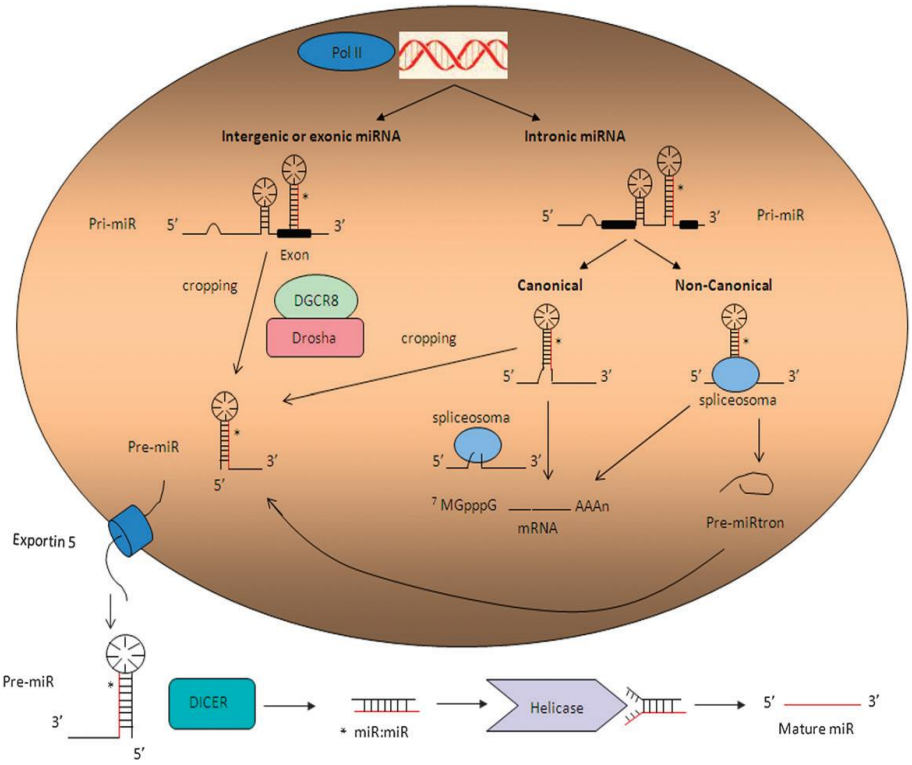

Figure 1. Intergenic, exonic and intronic miRNAs biogenesis [42].

1.2.2 miRNAs actions

miRNAs exert a complex and widespread regulation on its molecular targets.
miRNAs bind to complementary mRNA sequences, usually in their 3' untranslated region (3' UTR), which leads to mRNA degradation or to inhibition of protein translation. If there is complete complementation between the miRNA and target mRNA sequence, Ago2 can cleave the mRNA and lead to direct mRNA degradation. If there isn't complete complementation the silencing is achieved by preventing translation [43,44].
The mechanistic details of miRNA's function in repressing protein synthesis are not well understood. In addition, the results from studies conducted in different systems have often been contradictory. It is difficult to conclude whether the reported discrepancies are artifacts of different experimental approaches or whether miRNAs are indeed able to exert their repressive effects by disparate mechanisms.
miRNAs have been found to repress translation at initiation, either by targeting the cap recognition step or by inhibiting ribosome 80S complex assembly, but repression at postinitiation steps has also been reported [45-51].
Translationally silenced mRNAs are sequestered, away from the translational machinery, at distinct cytoplasmic sites, the so-called processing or P-bodies or GW bodies [52,53].
Subunits of miRISC (miRNAs, Argonaute proteins, and GW182) are also enriched in GW bodies [55,56]. At these locations mRNAs accumulate that are destined for storage or decay. Some P-body components are important for effective repression of protein synthesis by miRNAs [56,57].
A fraction of P bodies co-localizes with multivesicular bodies, membrane structures that play a role in miRNA mediated repression [58,59].
More recent studies have reported that miRNAs can also bind to the 5' UTR or the open reading frame and, even more surprisingly, they can upregulate translation [60-64].
In addition, it has been evidenced that mature miRNAs may also be localized in nucleus, through a specific hexanucleotide (AGUGUU) sequence which acts as a transferable nuclear localization element [65].
Figure 2 shows mature miRNA functions on target molecules.

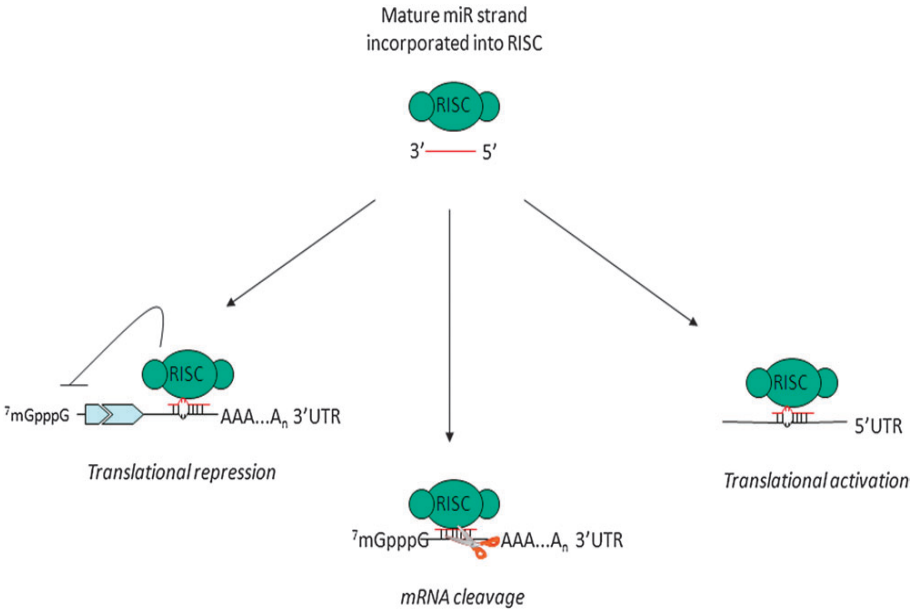

Figure 2. miRNAs gene expression regulation. Usually miRNAs block mRNA targets at post-transcriptional level by inhibiting their translation or stimulating degradation, but recently it has been reported an opposed role in up regulating translation [42].

1.2.3 miRNAs functions

miRNAs are one of the main genome regulators, targeting several hundreds of transcripts [66]. A single miRNA has several mRNA targets and, conversely, a transcript can be modulated by multiple miRs [67,68].

miRNAs are expressed in a tissue-specific manner and are involved in multiple physiological processes, mainly dealing with developmental and metabolic processes: cell proliferation, cell differentiation, development, cellular signaling, apoptosis, immune response, fat metabolism, insulin secretion, stem cell maintenance, neuronal patterning, haematopoietic lineage differentiation [69-77].

The first evidence that miRNAs might play a role in embryonic stem cells differentiation derives from the identification of embryonic stem cell-specific miRNAs in mouse [78]. miRNAs play a key role in the maintenance and differentiation of pluripotent embryonic stem cells [79], stabilizing the self-renewing versus differentiated cell fates [80] and playing a crucial role in germ layer specification [81].

Moreover it has been observed that miRNAs are not only required for the development of early embryonic stem cell survival and differentiation, but also play an important role in maintaining the survival of mature cells and their function. For example, in neurons miR-134 contributes to synaptic development, maturation and plasticity [82]. miR-133b is expressed specifically in midbrain dopaminergic neurons (DNs) and is deficient in midbrain tissue from patients with Parkinson's disease [83]. miR-133 is also considered as a muscle-specific miRNA that regulates muscle development [75].

miRNAs control oocyte maturation and corpus luteum development [84]; play important roles in differentiation and function of osteoclasts [85]; are specifically expressed in hematopoietic cells and their expression is dynamically regulated during early hematopoiesis and lineage commitment [72]; regulate glucose-induced insulin secretion and fat metabolism [73,86].

In the mammalian immune system miRNAs control has emerged as a critical regulatory principle. Genetic ablation of the miRNA machinery, as well as loss or deregulation of certain individual miRNAs, severely compromise immune development and can lead to immune disorders like autoimmunity and cancer [77].

Viruses use miRNAs in their effort to control their host cell; reciprocally, host cells use miRNAs to target essential viral functions. Experimental results have shown that miRNAs involved in innate immunity function as gene regulators and as a host cell defence against both RNA and DNA viruses [77,87]. The expression of host cell miR-122 can inhibit the replication of hepatitis C virus (HCV) and works through IFN-β [88]. The miRNA-silencing machinery controls HIV-1 replication, inhibiting virus replication in infected cells, in turn, HIV-1 actively suppresses the expression of the cluster miR 17-92, required for efficient viral replication [89].

Figure 3 summarizes miRNAs functions in physiological conditions.

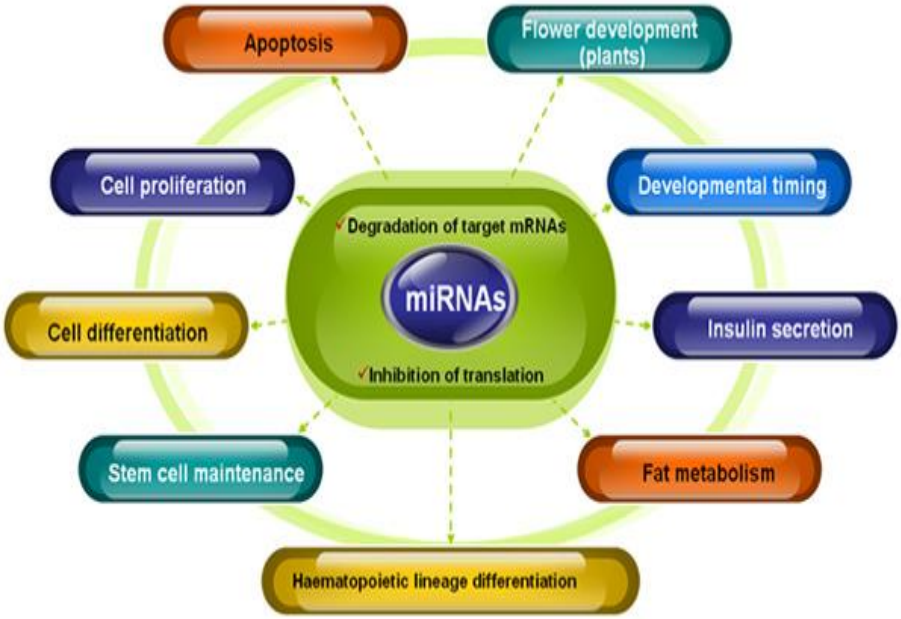

Figure 3. The roles of miRNAs in physiological conditions [90].

1.3 miRNAs and human cancers

As miRNAs are an integral part of the regulatory networks in cells, a perturbed miRNA function or altered miRNA expression may disorganise cellular processes and eventually cause or contribute to disease.
miRNAs aberrant expression has been reported in several types of diseases, including diabetes, cardiovascular disease and cancers [91-96].
A growing list of reports demonstrate that miRNAs play a critical role in cancer initiation and progression, as well in cancer cell aggressiveness, chemoresistance, radioresistance, migration and metastasis and that miRNA alterations are ubiquitous among human neoplasms. [97-101].
miRNAs can either modulate oncogenic or tumor suppressor pathways and their expression can be regulated by oncogenes or tumor suppressor genes [102].
An individual miRNA is capable of regulating dozens of distinct mRNAs [67,68], however, it remains unclear whether consequences upon miRNAs levels alteration depend on simultaneous deregulation of the entire repertoire of targets of a given miRNA or instead on the altered activity of only a small subset of effectors.
The first evidence of the involvement of miRNAs in human cancer derived from studies on chronic lymphocitic leukemia. Cytogenetic studies indicated deletions at chromosome 13q14 in approximatively 50% of chronic lymphocitic leukemias, and the critical region did not contain any protein coding tumor suppressor gene but two miR genes, miR-15a and miR-16–1, that are expressed in the same polycistronic RNA. This result evidenced that the deletion of chromosome 13q14 caused the loss of these two miRNAs and this event could be involved in the pathogenesis of human cancer [103].
After these early studies indicating the role of miRNAs in the pathogenesis of human cancer, platforms to assess the global expression of miRNAs genes in normal and diseased tissues have been developed, as an effort to establish whether miRNA profiling could be used for tumor classification, diagnosis and prognosis [104].
miRNAs profiles can distinguish between normal and cancerous tissue, identify tissues of origin, discriminate different subtypes of a particular cancer or even specific oncogenic abnormalities [105,106].
Even more importantly, miRNAs profiling can predict disease outcome or response to therapy [107-111].
Also the possibility to evaluate miRNAs expression to predict the response to specific drugs might be useful for a more accurate selection of patients potentially responsive to a specific therapy [111,112].
The predictive and prognostic values of miRNAs signatures have been validated for several types of tumors. miRNAs small size contributes to a higher stability in comparison with mRNAs, allowing the study of their expression in fixed tissues or other biological material, and thus supporting their possible use as novel, minimally invasive and robust biomarkers.
miRNAs can be extracted and detected from paraffin-embedded tissues, from blood (either total blood, plasma or serum) [113] and from circulating exosomes [114].
Moreover, it has been reported that the profile of circulating miRNAs of individuals affected by different neoplasms reflects the pattern observed in the tumor tissues,

suggesting the possibility of using circulating miRNAs as easily detectable tumor biomarkers [115].
Recently, was identified a miRNA expression signatures with strong predictive, diagnostic, and prognostic potential analyzing plasma samples of lung cancer patients collected 1–2 years before the onset of disease [116].

1.3.1 miRNAs function as oncogenes or tumor suppressors

Some miRNAs are down regulated in various human cancers, which suggests that they may function as tumor suppressors. Tumor suppressor miRNAs usually prevent tumor development by negatively inhibiting oncogenes and/or genes that control cell differentiation or apoptosis. Currently, several miRNAs are considered as tumor suppressor genes, for example, miR let-7.
the let-7 family of miRNAs is downregulated in many tumors, including lung and breast cancer [108,117,118].
In lung cancer patients reduced let-7 expression is significantly associated with shortened post-operative survival, independent of disease stage [119].
let-7 family members functionally inhibit the mRNAs of well-characterized oncogenes, such as the Ras family [118], HMGA2 [120], and c-myc [121].
miR-15 and miR-16 are down regulated in chronic lymphocytic leukemia and target an anti apoptotic gene BCL2 [122].
Colon cancer is also associated with alteration in miRNAs expression. miR-143 and miR-145 are down-regulated at the adenomatous and cancer stages of colorectal neoplasia [123].
On the other hand, some miRNAs genes are over-expressed in cancers, indicating that they may have roles as oncogenes and accelerate the development of cancer.
These miRNAs usually promote tumor development by negatively inhibiting tumor suppressor genes and/or genes that control cell differentiation or apoptosis.
Members of the miR 17-92 cluster are highly expressed in a variety of solid tumors and hematological malignancies, including cancers of the breast, colon, lung, pancreas, prostate, and stomach as well as lymphomas [124-126]. These miRNAs promote proliferation, inhibit apoptosis, induce tumor angiogenesis. Among the experimentally validated targets of miR 17-92 cluster members is the transcription factor c-myc, important regulator of cell growth often mutated or amplified in human cancers, the tumor suppressors PTEN and p21, and E2F transcription factors, critical regulators of the cell cycle [127-129].
In several types of lymphomas, derived from B cells of different developmental stages, the expression of miR-155 is increased compared to normal cells [130].
One possible mechanism for miR-155 involvement in this type of cancer is due to the down-regulation of the expression of the transcription factor PU.1, which is required for later differentiation of B cells [131]. Moreover, recent studies showed direct involvement of miR-155 in regulation of PI3K-AKT pathway, a major mediator of cell survival through direct inhibition of pro-apoptotic signals, in diffuse large B cell lymphomas [132].
miR-155 is also reported to be over-expressed in several types of human solid tumors including breast, colon, and lung cancer [108,117,126].
More recently it has been demonstrated that a miRNAs can act as oncogenes o tumor suppressors according to the cellular context of its target genes [133-136].

1.3.2 miRNAs in cancer invasion and metastasis

Tumor metastasis is a significant factor in the clinical management of cancer, as most cancer mortality is associated with disseminated disease rather than the primary tumor [137].

Metastasis is a complex, multi-step process: primary tumor cells invade adjacent tissue, enter the systemic circulation (intravasate), translocate through the vasculature, arrest in distant capillaries, extravasate into the surrounding tissues and finally proliferate from initial microscopic growths (micrometastases) into macroscopic secondary tumors [138].

The traditional view of metastasis includes the process of clonal selection in which variant clones within the primary tumor become capable of completing the complex multistep metastatic process [138,139].

Recently, expression profiling analyses have revealed various tumor metastasis genes and metastasis suppressor genes, which not only regulate the metastatic process [140-148] but also maintain the microenvironment of tumor cells, and initiate the process of epithelial–mesenchymal transition (EMT), characterized by loss of cell adhesion, repression of E-cadherin expression and increased cell motility.

These metastasis-associated genes have important roles in tumor invasion and metastasis, and miRNAs networks act as upstream regulators of these genes in tumorigenesis and metastasis. An increasing number of pro-metastatic miRNAs and antimetastatic miRNAs from miRNAs profiling of tumor versus non-tumor tissues have been identified as regulatory inhibitors of metastasis genes or metastasis suppressor genes**.**

miR-10b was reported to promote breast tumor metastasis [100], and subsequently was found to participate in a complex molecular pathway involving metastasis-related genes, such as TWIST and the breast cancer metastasis suppressor 1 (BRMS1) [149].

Previous studies have shown that miR-21 expression is increased in many solid tumors, such as glioblastoma [150], breast [117], lung, prostate, colon and stomach cancer [126]. But miR-21 seems to produce a more marked effect on tumor metastasis. The level of miR-21 expression correlates significantly with advanced clinical stage, metastasis and poor prognosis in these tumors [151].

Moreover, miR-21 was found to stimulate cell invasion and metastasis in different tumor models (breast and colon cancer, or gliomas) both in vitro and in vivo [152-154].

Many targets of miR-21 have been identified by bioinformatic prediction and molecular biological assay, which include some metastasis-associated genes directly regulating tumor metastasis. For example Tropomyosin 1, an actin-binding protein, promotes cell transformation and tumor metastasis when down-regulated by miR-21 [155,156].

Programmed cell death 4 (PDCD4) is a another important target of miR-21, experimentally validated in colon cancer [152], breast cancer [154,157,158] and cholangiocarcinoma [159], which negatively regulates invasion and metastasis.

PDCD4 can by itself inhibit miR-21 [160], evidencing complex reciprocal regulatory networks between miRNAs and target genes.

Also several tumor metastasis-associated genes, as Transforming Growth Factor β (TGF-β) or Epidermal Growth Factor (EGF), exert a role in regulating miR- 21 expression [153,161].
miR-373 and miR-520c, which belong to the same miRNA family, have been identified as metastasis promoting miRNAs [162]. miR-373 suppressed the oncogene-induced p53 pathway and cooperated with oncogenic RAS to promote cellular transformation [163].
Another tumor metastasis-promoting miRNA, miR-182, was recently identified in melanoma [164].
miR-183 was identified as a negative regulator of lung cancer metastasis [165]. The target gene, Ezrin, has a role in controlling the actin cytoskeleton, cell adhesion and motility.
miR-335, miR-126 and miR-206 were reported as potential metastasis suppressors in human breast cancer [166]. The miRNAs were consistently down-regulated in metastatic foci, and restoring their expression significantly decreased the number of metastatic foci. The low miR-335 or miR-126 expression in human primary tumors was significantly associated with poor metastasis-free survival.
The downregulation of miR-126 was also found in lung cancer [108].
miR-206 can activate apoptosis, and inhibit cancer cell migration and foci formation by targeting NOTCH3 [167].
The let-7 miRNA family is one of the tumor suppressing miRNAs that can inhibit both tumorigenesis and metastasis. Several important oncogenes contributing to EMT have been identified as the targets of let-7 [118,127,168].

1.3.3 miRNAs deregulation in human cancers

Alterations in miRNAs expression may promote tumour formation by modulating the functional expression of critical genes involved in tumour cell proliferation or survival. Many changes occur in cancer cells that, in a direct or indirect manner, may influence miRNA expression, e.g. genomic rearrangements, abnormalities in miRNAs processing genes or proteins, and the disrupted epigenetic regulation of miRNAs.
Several mechanisms can control miRNAs expression and result to be altered in human diseases.
Structural genetic alterations can affect miRNAs expression: chromosomal abnormalities, mutations, or single nucleotide polymorphisms, as first suggested by the evidence that miRNAs are frequently located in regions of the genome involved in cancer alterations, such as deletion or amplification.
Indeed, chromosomal regions encompassing miRNAs involved in the negative regulation of a known tumor suppressor may be amplified. This amplification would result in the increased expression of the miRNA and the consequent silencing of the tumor suppressor gene. Viceversa, miRNAs able to inhibit oncogenes are often located in fragile regions of the genome, where deletions or mutations can be responsible for their reduced levels and the resulting overexpression of the target oncogene [103,169,170].
In addition, the deregulated miRNAs expression can also be due to epigenetic changes, as altered DNA methylation, suggested by the evidence that half of the genomic sequences of miRNAs genes are associated with CpG islands [171]. Another epigenetic mechanism is represented by histone acetylation, in fact histone deacetylase inhibition rapidly alters miRNAs levels [172,173].

Furthermore miRNAs themselves are able to regulate the expression of components of the epigenetic machinery [174,175].
Defects in the miRNAs biogenesis machinery can also have consequence on miRNAs expression, in particular, it seems that Dicer or Drosha silencing promotes cellular transformation and tumorigenesis in vivo and has also been inversely correlated with outcome in lung, ovary and breast cancers [176-178].
miRNAs processing can be also affected by other miRNAs, directly or indirectly, thus creating a complex level of reciprocal interaction and regulation [179,180].
Finally, a deregulation of miRNAs expression can be a result of increased or decreased transcription due to an altered transcription factor activity [181-183].

1.3.4 miRNAs and cancer therapy

It was only 10 years ago that the first human miRNA was discovered, and yet a miRNA-based therapeutic has already entered Phase 2 clinical trials.
MiRNAs have rapidly emerged as promising targets for the development of novel anticancer therapeutics. The development of miRNA-based cancer therapeutics relies on restoring the activity of tumor suppressor miRNAs using double-stranded miRNA mimics or inhibition of oncogenic miRNAs using single-stranded antisense oligonucleotides, termed anti-miRNAs.
The most advanced candidates are mimics of the let-7 and miR-34 to target a broad spectrum of solid tumors.
Target repressed by let-7 is KRAS, an oncogene frequently mutated in lung cancer and other cancer types [118,184].
Systemic delivery of a miR-34 mimic blocked tumour growth in mouse models of lung and prostate cancer reducing proliferation and enhancing apoptotic activity of tumour cells, by a specific repression of CDK4, Met and BCL2 [185].
miRNA-based therapeutic that is most clinically advanced at the present time targets an infectious disease, hepatitis C (HCV). Specifically, a miR-122 antagonist, blocks replication of the virus [186].

1.3.5 miRNAs and breast cancer

One of the first solid tumors to be profiled for miRNA expression was, in 2005, breast cancer [117].
Analysis of miRNAs expression patterns in breast cancers has led to the identification of signatures which can differentiate tumor from normal tissue [106,117].
The first miRNA signature characteristic of breast carcinoma identified 13 miRNAs able to discriminate tumors and normal tissues with an accuracy of 100%.
Among the most significant miRNAs differentially expressed, miR-21, over-expressed in breast cancers, has been demonstrated to mediate cell survival and proliferation directly targeting the onco-suppressor genes (for example PTEN), and it has been associated with advanced clinical stage, lymph node metastasis and patient poor prognosis [187,188].
miR-21 has been also detected freely present in the circulation, and high circulating concentrations correlated significantly with visceral metastasis [189,190].
These data evidence a role of miR-21 not only in tumor growth but also in invasion and metastasis.
Conversely, down regulated miRNAs, as miR-125a and b and miR-205, regulate oncogenes, as tyrosine kinase receptor HER-2, playing important role in cellular

proliferation and survival. Cells overexpressing miR-125a or miR-125b are impaired in their anchorage-dependent growth, migration, and invasion capacities [191,192].
miR-145 is another miRNA reported to be down-regulated in breast cancer cells and tissues, and to decrease cell proliferation and invasion through silencing of genes such as Fascin 1 (an Actin-bundling protein, important for the formation of cell protrusions), c-myc (regulator of cell growth), Mucin 1 (a cell surface associated mucin, with cell signaling properties), and SMAD2/3 (intracellular proteins transducing extracellular signals that activate TGFβ gene transcription) [193].
Analysis of the messenger RNA targets of miRNAs with differential expression in normal and tumor breast tissues indicates that their aberrant expression impacts the regulation of important cellular networks known to drive breast cancer [194]. This is supported by the observation that several clinically relevant breast tumor features, such as tumor size, nodal involvement, vascular invasion, hormone receptor and Her-2 status, are also related to the expression of particular miRNAs [106,117,195].
In particular, was identified a panel of miRNAs differentially expressed in Estrogen Receptor (ER) positive versus ER negative breast carcinoma patients, being miR-191 and miR-26 the most significantly over-expressed, and miR-206 the most significantly down-modulated [117].
A recent study also reported an inverse correlation between miR-155 expression levels and estrogen and progesterone receptors expression in breast cancer patients. miR-155 is known to be overexpressed in several types of cancer, included mammary carcinomas, and is a potent suppressor of apoptosis and cell cycle arrest via the p53 network inhibition [196].
miRNAs profiling highlighted novel potential predictive indicators, which may contribute to improve selection of patients for specific therapies. For example, over-expression of miR-221 and miR-222 is responsible for resistance to anti-estrogenic therapies [197,198].
It has also been demonstrated that miRNAs exert a crucial role not only in controlling the primary tumor growth by regulating pathways involved in cell cycle and proliferation, but also in modulating migration, invasion and the interaction with the microenvironment, mechanisms related to the acquisition of a more aggressive phenotype and promoting the onset of the metastatic process.
Expression of miR-10b and loss of expression of miR-126 and miR-335 are causally linked to the development of breast cancer metastasis [100,166].
miR-126 and miR-335 are two miRNAs specifically lost as human breast cancer cells develop metastatic potential and are down-regulated in the majority of primary breast tumours from patients who relapse correlating with poor distant metastasis-free survival.
Restoring the expression of these miRNAs in malignant cells suppresses lung and bone metastasis by human cancer cells in vivo. miR-126 restoration reduces overall tumour growth and proliferation, whereas miR-335 inhibits metastatic cell invasion.
miR-335 suppresses metastasis and migration through targeting of the transcription factor SOX4, a regulator of cell progenitors development and migration, and extracellular matrix component tenascin C [166].
miR-10b is a crucial downstream effector of TWIST1, a transcription factor known

to induce epithelial-mesenchymal transition [100].
miR-335 has also recently found deleted or epigenetically silenced in breast cancer metastasis [199].
Evidences from several studies suggest that the miR-200 family plays a crucial role in regulation of breast cancer metastasis and aggressiveness. miR-200 family targets specific molecular markers of EMT, including E-cadherin, marker of epithelial phenotype, vimentin, ZEB1 and ZEB2, markers of mesenchymal phenotype. This family of miRNAs stimulates epithelial phenotype by suppressing the expression of EMT inducers, such as ZEB1 and ZEB2. Conversely, EMT induction by TGFβ, a well known cytokine promoting this process, represses miR-200 family members and stimulate ZEB1 and ZEB2 expression [200,201].
Hence miRNAs might be used as markers of the metastatic potential of primary breast tumors.

1.4 miR-296

We previously identified loss of miR-296 expression during tumorigenesis in a well established model of pancreatic tumor progression (RIP-TAg transgenic mice), in several human cultured-tumor cell lines and in a series of different human carcinoma samples from formalin fixed and paraffin embedded specimens, compared to normal tissues [202].
miR-296 (chr. 20q13.32) plays important roles in different cell types and cellular pathways, regulating several distinct mRNAs. The more recent observations indicate that miR-296, along with miR-298, is part of the GNAS complex locus, a highly complex cluster with imprinted gene expression, coding a stimulatory G-protein alpha subunit (Gs-α), involved in many signal transduction pathways [203].
miR-296 was initially found to be specifically expressed in differentiated mouse embryonic stem cells, directly cross-talking with Nanog, Oct4 and Sox2 genes [78,204] and subsequently it was characterized in human embryonic stem cells [205,206].
miR-296 was also observed to be involved in antiviral responses induced by IFNα/β, inhibiting HCV replication directly targeting viral transcripts [88].
Furthermore miR-296 was identified in endothelial cells of normal and neoplastic tissues, where it promoted angiogenesis through inhibition of one of its target gene, the hepatocyte growth factor-regulated tyrosine kinase substrate (HGS). HGS normally stimulates degradation of growth factors receptors, such as vascular endothelial receptor-2 (VEGFR2) and Platelet derived growth factor receptor β (PDGFR-β) [207].
Finally, in a large series of human cancer cell lines and carcinoma specimens miR-296 was identified as a comprehensive regulator of cell tumorigenicity, migration, and invasion [202], by inhibition of the expression of one of its targets, Scrib, a cytoplasmic protein that participates in multiprotein complexes.

1.4.1 miR-296 and cancer

In tumors the function of miR-296 is not fully elucidated.
In squamous cell carcinomas of the esophagus miR-296 is reported to be over-expressed and to have a pro-tumorigenic role. High levels of this miRNA are associated with resistance to chemotherapy, while its forced down-regulation resulted in increased sensitivity to standard chemotherapeutic agents and in decreased tumorigenesis of esophageal carcinoma cell lines, probably through reduction of cyclin D1 and upregulation of p27 [208,209].
Based on these data, determination of miR-296 levels from a diagnostic biopsy specimen has been proposed to assess patients who benefit from neo-adjuvant therapy [210].
In hepatocarcinomas, a reduced expression of miR-296 correlates with decreased survival [211] and in prostate cancers low levels are associated with an advanced stage and a high grade of malignancy [212].
miRNAs profiling in patients with advanced bladder cancer treated with standard chemotherapy identified a panel of 15 miRNAs differentially expressed between patients who experienced progression of disease and those who had complete responses. miR-296 was found within this subgroup of miRNAs to be down-regulated in patients with a progressive disease [213].

In colorectal cancer, miR-296 plasma levels decrease is associated with shorter survival, more aggressive tumor phenotype and increased rate of metastasis [214].

1.4.2 miR-296 and breast cancer

In breast cancer available data are not numerous.
We analyzed miR-296 expression levels in various human tumor cell lines, including breast, and in primary breast carcinomas as well as in their distant metastases. Our observations revealed a profound repression of miR-296 levels in tumors compared with normal mammary epithelial cells and tissues. Furthermore manipulation of miR-296 levels in human breast cancer cell lines evidenced enhanced tumor cell migration and invasion when miR-296 was depleted, conversely, its forced re-expression suppressed tumor growth in xenograft models [202].
One recent study explored the miRNA expression profile in a subset of primary breast cancers, defined as “triple negative tumors” (i.e. negative for estrogen and progesterone receptors and for Her-2 expression), associated with a poorer prognosis. Expression of miR-296 did not exhibit any significant difference between breast cancer and normal mammary tissue. Relationships between miR-296 expression and pathologic features of tumors and influences on patients overall and disease free survival were not observed [215].

1.5 Scrib

Breast cancer is thought to originate from epithelial cells of the terminal ductal lobular units in the breast [216]. Each terminal ductal lobular unit has multiple small units referred to as acini that consist of a single polarized layer of luminal epithelial cells surrounding a hollow lumen [216]. Polarity is defined by distinct apical and basolateral membrane domains separated by intercellular tight junctions. The establishment and maintenance of polarized organization is critical for normal function of mammary epithelial cells in vivo.
Early during initiation and progression of carcinoma, epithelial cells loose their ability to maintain a normal polarized organization suggesting a critical role for molecules that regulate cell polarity in breast cancer.
Establishment of apical-basal polarity in mammalian epithelia is coordinated by a set of proteins referred to as polarity regulators [217]. These include the Scrib/Lethal (2) giant larvae (Lgl)/Discs large (Dlg) proteins that direct formation of basolateral membranes. The Crumbs/ protein associated with Lin7(PALS)/ tight-junction associated PDZ protein (PATJ) and Partitioning defective 3 (Par3)/Par6/atypical protein kinase C (aPKC) protein complexes that direct establishment of the apical membrane and the apical-basal border, respectively [217-219].
We recently reported that miR-296 is involved in control of cell tumorigenic potential and of cell motility by blocking the expression of one of these polarity regulators, its target Scrib [202].
Scrib belongs to the LAP (LRR and PDZ) protein family, containing leucine-rich domains (LRR), involved in the formation of protein-protein interaction, and PDZ domains (PSD95, Dlg-1 and Zo-1 proteins) that anchor transmembrane proteins to cytoskeleton and hold together signaling complexes [220].
In epithelial tissues, Scrib is recruited to sites of cells contact where it associates with junctional proteins for maintenance of apico-basal cell polarity and three-dimensional tissue organization [221-223].
In motile cells, Scrib participates to directional cell motility, via the assembly of multiprotein complexes and the activation of small GTPase signaling at the leading edge of migrating cells [224-228].
Accordingly, loss-of-function of Scrib in mutants [229] or depletion of Scrib [226] disrupted apical-basal polarity [230] and cooperated with oncogenic signals [231] to enhance tumor cell migration, invasion, and survival [232].

1.5.1 Scrib and cancer

Analyses of Scrib expression in tumors have produced somewhat contradictory results. Initial evidences suggested that Scrib expression was lost during human tumorigenesis, while other reports have shown that Scrib levels were increased. [202,232-240].
Early studies have proposed a model of Scrib function in an evolutionary-conserved pathway of tumor suppression [220], with alteration of cell polarity and increased tumor cell migration in association with Scrib loss [230,233].
Recent reports have shown that Scrib levels are increased and subcellularly mislocalized, from cell-cell contacts into cytoplasm, in at least certain human tumors [236,237].

These data indicate a complex setting, in which aberrant over-expression of Scrib in tumors, coupled to a subcellular mislocalization, disrupts cell motility pathways, favoring random cell migration, and contributing to the acquisition of a migratory cellular phenotype [227,230,237].
High-resolution genomic analyses in breast and ovarian cancers showed that Scrib locus, on 8q24.3, was often amplified [234,235].
In colorectal adenomas and adenocarcinomas accumulation of Scrib protein was observed in comparison with the adiacent normal epithelium. Moreover, loss of Scrib membranous staining was detected in association to the cytoplasmic translocation of β-catenin, suggesting a role in colon carcinogenesis [236].
We showed that Scrib is globally over-expressed in cultured tumor cell lines and in cancer patient series representative of different human neoplasms (malignant mesothelioma, cancers of colon, breast, lung, stomach, uterus, prostate, liver, ovary, bladder, central nervous system) compared with matched normal tissues. In tumor cell lines and tissue specimens Scrib was also found predominantly in the cytoplasm instead of a membrane localization, as seen in normal epithelia [237].
We also found that Scrib protein was abundantly over-expressed in different primary and metastatic epithelial malignancies, correlating with shortened overall survival in hepatocellular carcinoma patients [202].
Furthermore, results obtained from silencing of Scrib in a model of lung cancer cells evidenced impaired tumor cell migration and invasion and down-regulation of several markers of cell motility and EMT [237].
Conversely, histochemical analysis showed a dramatic decrease in the expression of Scrib with the progression of disease from normal uterine cervical tissues to invasive cervical cancers through the precursor lesions of human papilloma virus (HPV) infected patients. Quantitative PCR revealed a clear Scrib down-regulation in the invasive uterine cancers, but not in high grade precursor lesions. Scrib is a substrate of ubiquitin-mediated degradation by human papillomavirus E6 protein and the host E6AP ubiquitin-protein ligase. Degradation HPV-mediated may be one of the causal roles for the progressive decrease of Scrib expression during the disease progression from low-grade to high grade squamous intraepithelial lesions. Cooperative role of down-regulation of Scrib mRNA expression driven by unknown factors and ubiquitin-mediated degradation of Scrib led to the complete decrease of Scrib expression during the process of carcinogenesis from high grade squamous intraepithelial lesions to invasive cancer [238].
Deregulation of Scrib was also described in prostate cancer. Biallelic loss of Scrib in mice was sufficient to cause low grade prostate intraepithelial neoplasia. Loss of cell polarity and increased proliferation were concomitant with increased Ras/MAPK signaling in Scrib deficient prostate lesions, suggesting a negative regulation of Scrib on this pathway to suppress prostate tumorigenesis. These data indicated that Scrib deficiency is involved in initiation of prostate cancer and that additional oncogenic or tumor suppressor mutations are required for Scrib deregulation to contribute to tumor progression. According with these observations, Scrib deregulation and mislocalization in human prostate cancers strongly correlated with poor survival [239]

1.5.2 Scrib and breast cancer

Loss of Scrib in mammary epithelia disrupted cell polarity, blocked three-dimensional morphogenesis, inhibited apoptosis and induced neoplastic growth.

Mislocalization of Scrib from cell-cell junction was also sufficient to promote cell transformation [233].
Data from human breast cancer cell lines showed inhibition of cells migration and invasion, and wrong cellular orientation towards the leading edge of migration in association with Scrib forced depletion [202] and a decreased growth of cancer cells in xenograft assays [240].
In a series of human breast cancers Scrib was ubiquitously over-expressed in tumors compared with normal matched tissues irrespective of hormonal receptor and Her-2 status. Although Scrib was detectable in normal mammary glands with a membranous distribution at cell-cell junctions, tumor-associate Scrib was subcellularly mislocalized and exhibited a predominantly diffuse cytoplasmic reactivity [237].
Gene expression study revealed poor clinical outcome in human breast cancers in association with augmented Scrib transcripts, while reduction of its levels by small interfering RNA experiments slowed the growth of human breast cancer cells in mouse xenografts [240].
Breast cancers showed also changes in Scrib protein localization respect to normal mammary parenchyma, from sites of cell–cell contact to a diffuse cytoplasmic or a combination of membranous and cytoplasmic staining in primary and metastatic tumors [202,240]
Emerging data suggest that probably functions of Scrib are context-dependent, and related to changes in multiprotein-complexes with which Scrib associates to regulate cell polarity and motility and finally cancer progression [240].

2. AIM OF THE STUDY

miRNAs are a class of small non-coding RNA molecules that regulate gene expression by directly targeting mRNA. They have been implicated in several human cancers, given their role in numerous physiological processes usually deregulated in tumors.
miR-296 and one of its best characterized targets, Scrib, are involved in control of cell plasticity and motility, and are reported to be deregulated in human cancers.
We aimed to investigate the expression levels of miR-296 and its target Scrib in a large series of well clinically characterized primary breast cancers, nodal and distant metastases.
Objectives of this study were to identify any correlations of this miRNA and its target with clinical and pathological parameters, useful for prognostic and/or predictive purposes and to gain knowledge about their role in mammary tumorigenesis.
Furthermore, to evaluate whether modifications of miR-296 levels could have effects on neoplastic growth, and therefore modulation of miR-296 may represent a new therapeutic option for breast cancer patients, we injected human breast cancer cells in mice, subsequently administered to them pre-miR-296, and measured cancer growth over time.
Our observations may contribute to elucidate molecular pathways involved in breast cancer development and dissemination and to identify potential targets for tailored therapies.

3. MATERIALS AND METHODS

3.1 Patients

We evaluated a unselected cohort of 198 patients with invasive breast cancer, surgically resected between 1985 and 2006 at the San Paolo Hospital in Milan. Formalin fixed and paraffin embedded tissue samples were available for all of the patients. Each case was reviewed in order to evaluate the histological type, tumor grade, presence of lymph node metastases, and categorized according to the TNM classification 2009 [241].

The histological grade was determined according to criteria reported by the Nottingham modification of Bloom and Richardson score [242].

Of 198 cases, 131 infiltrating tumor samples had adequate material for concurrent molecular and immunohistochemical analysis. Thirty-one in situ carcinomas and 35 lymph node metastasis out of 131 patients were obtained. Small amount of available material in most specimens for these lesions allowed us to perform only immunohistochemical analyses.

For twenty-one patients sufficient material for molecular analyses from distant metastatic lesions was available beside primary tumor. Scrib immunohistochemistry for these subset of samples was performed on full sections.

Due to the frequent fibro-adipose involution of the normal breast parenchyma, 77 cases out of 131 had representative normal mammary lobules for contemporary analysis of miR-296 and Scrib.

The median age at diagnosis was 62 years (range 33-89 years). There were 50 deaths, with a disease-free survival (DFS) between 0 and 300 months (mean 121 months) and an overall survival (OS) between 3 and 300 months (mean 136 months).

The study was approved by an Institutional Review Board of University of Milan, School of Medicine.

Clinical and pathological characteristics of patients are summarized in Table 1.

HISTOTYPE	***N.OF PATIENTS***
Ductal infiltrating carcinoma	114 (87%)
Lobular infiltrating carcinoma	16 (12,2%)
Mixed ductal-lobular carcinoma	1 (0,8%)
GRADE	
G1	14 (10,7%)
G2	76 (58%)
G3	41 (31,3%)
pTNM	
pT1	73 (55,7%)
pT2	50 (38,2%)
pT3	3 (2,3%)
pT4	4 (3%)
na	1 (0,8%)
pN0	59 (45,1%)
pN1	35 (26,7%)
pN2	17 (13%)
pN3	10 (7,6%)
na	10 (7,6%)
M0	86 (65,6%)
M1	45 (34,4%)
METASTASIS SITE	
Only bone	20 (15,3%)
Only visceral	18 (13,7%)
Bone and visceral	7 (5,3%)

Table 1. Characteristics of 131 infiltrating breast cancers available for concurrent molecular and immunohistochemical analysis.

3.2 Tissue micro-array (TMA) construction

In TMA technique, cylindrical tissue samples are taken from many different archival tissue blocks and placed into one empty 'recipient' paraffin block.
Sections from TMA blocks can be used for simultaneous evaluation of hundreds or thousands specimens, appliying all different types of in situ tissue analyses (immunohistochemistry and/or in situ hybridization).
As demonstrated by multiple studies, results obtained on TMA are highly representative of their donor tissues, despite the small size of the individual specimens.
Moreover, small diameter of the specimen taken out of the donor block maximize the number of samples that can be taken from one block and minimizes the tissue damage inferred to the donor block, leaving punched tissue blocks fully interpretable for analyses that may subsequently become necessary [243].
TMA was constructed as previously detailed [244], with slight modifications.
Routinely formalin fixed paraffin-embedded tissue samples were used.
For each case, areas of interest were identified by morphological assessment on histological sections stained with hematoxylin-eosin and marked on the slide.
Archival blocks corresponding to the slides examined were recovered and the presence of adequate amounts of material was assessed.
A tissue cylinder (core) was punched from a histologically representative area of each 'donor' block, and then inserted into an empty 'recipient' paraffin block using the tissue arrayer Tissue Arrayer MiniCore (Alphelys, Plaisir, FR)
In order to ensure a satisfactory quantity of material in each case, we used cores of 1 mm in diameter.
We select 4 cores for each infiltrating tumour case, 1 core of in situ carcinoma, 1 core of lymph node metastasis and 2 cores of normal mammary parenchyma to build the 'receiving' TMA block.
Figure 4 illustrates a TMA construction.

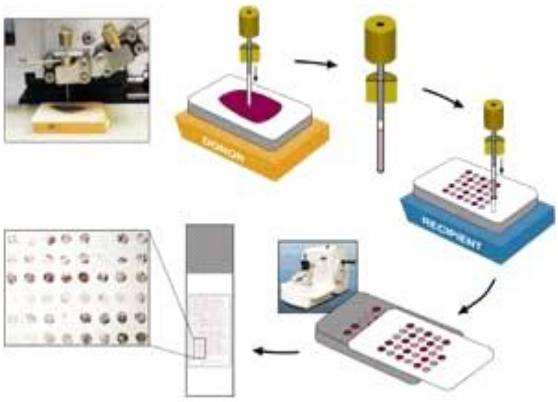

Figure 4. Steps of TMA construction, from ‘donor’ block to ‘receiving’ block and the subsequent slide.

3.3 Molecular analysis

At the same time, samples for miR-296 expression evaluation in infiltrating breast cancers and in normal counterparts were obtained from all patients who displayed satisfactory RNA quality and quantity (absorbance ratio: 1.2-2; total RNA≥100ng).
To this end a core of infiltrating tumor and one of normal breast parenchyma were collected in test tubes (RNase-free, Biopure, Eppendorf) and subjected to extraction of total RNA using MasterPure™ RNA Purification Kit (Epicentre Biotechnologies, Madison, Wisconsin, USA).
Available distant metastatic lesions were laser-assisted microdissected (Leica LMD6000, Leica Microsystems, Milan, Italy) and subjected to RNA purification as before.
For each sample, 100 ng of total RNA were reverse transcribed, using primers specific for miR-296 and RNU48 as reference gene (TaqMan® MicroRNA Reverse Transcription kit, Applied Biosystems by Life Technologies Corporation, Paisley, UK).
Finally, a Real Time-PCR analysis was performed with primers and probes specific for the target and reference small RNAs (hsa-miR-296-5p assay ID 527 and RNU48 assay ID 1006, TaqMan® MicroRNA Assay, Applied Biosystems by Life Technologies Corporation, Paisley, UK).
The expression of miR-296 relative to the reference gene (RNU48) was obtained applying the $2^{-\Delta Ct}$ formula. Raw data (Ct value) were converted into miR relative quantities (RQ) using the median expression value of miR-296 in breast cancers and normal mammary parenchyma as normalization factor.
When the 21 patients with distant metastases were analyzed, miR-296 expression in normal parenchyma was set equal to the unit for each sample.

3.4 Immunohistochemical analysis

Sections of 4-μm thick were cut from TMA or full-section blocks and stained with a goat polyclonal antibody specific to Scrib (clone C-20, Santa Cruz Biotechnologies, Santa Cruz, California, USA), as previously reported [237].
For antigen retrieval, slides were microwaved for 35 minutes incubated for 1 hour at 22°C in citrate solution.
Immunohistochemical staining for estrogen receptor (clone 1D5, Dako, Glostrup, Denmark), progesterone receptor (clone 636, Dako, Glostrup, Denmark), Her-2 (polyclonal, Dako, Glostrup, Denmark) and Ki-67 (clone MIB-1, Dako, Glostrup, Denmark) were made in order to phenotypically characterize tumors.
For the immunohistochemistry the automatic staining BioGenex i6000 Automated Staining System (BioGenex) was used.
The reactions were detected by Novolink Polymer Detection System (Novocastra Laboratories Ltd.), according to the manufacturer's instructions.
All slides were counterstained with hematoxylin.
Negative controls were incubated in the absence of primary antibody.
The Scrib cytoplasmic and/or membranous immunoreactivity was evaluated with a semi-quantitative method, as follows: negative (0), weak (1), moderate (2), or intense (3).
Estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (Her-2) were assessed according to the American Society of Clinical Oncology (ASCO)/College of American Pathologists (CAP) guidelines [245,246].
Ki-67 expression was quantified counting stained cells among the total number of carcinoma cells determined in 10 fields of infiltrating breast cancer cores, at a magnification of 400x, and expressed as a percentage [247].
Immunostained slides were evaluated by light microscopy (Leica DMLS, Leica Microsystems, Milan, Italy).

3.5 MiR-296 levels restoration analysis

All experiments involving animals were approved by an Institutional Animal Care and Use Committee.
Xenografts were generated injecting $2x10^6$ MDA-MB231 cells subcutaneously in the fourth mammary fat pad of 10 female, 6 weeks old, CD1 athymic mice (Charles River Laboratories International, Inc., Wilmington, Massachusetts, USA).
Tumor growth was monitored externally using Vernier caliper and tumor volume was calculated applying the formula: $V= (width)^2 \times lenght/2$.
When tumor reached a volume of about 50 mm^3, we injected directly in the tumor mass 3 nmol of control pre-miR (AM17110, Ambion, Life Technologies Corporation, Paisley, UK) or of pre-miR-296-5p (PM10609, Ambion, Life Technologies Corporation, Paisley, UK), complexed with 100 μL of AteloGene™ (KOKEN Co.,Ltd, Tokio, Japan).
Four animals were treated with control pre-miR, and six with pre-miR-296-5p.
Pre-miR injection day was considered as time zero (T0).
A second administration of miR molecules was performed after seven days and mice were sacrificed at day 14.
Tumor size was measured every day.
To normalize tumor volume data, each daily measurement was related to corresponding T0 volume.
Excided xenografts were formalin-fixed and paraffin-embedded following standard pathology procedures.

3.6 Statistical analysis

Real Time PCR experiments were performed in duplicate.
Differences in miR-296 expression between neoplastic and normal parenchyma samples were analyzed using the Student's t test.
To compare Scrib tissue expression levels in breast cancers and in healthy mammary parenchyma Mann Whitney U test was applied.
Samples classification into categories was assessed by Chi square test.
Data for overall survival and disease-free survival were analyzed in relation to the expression of miR-296 or its target Scrib.
In each analysis, patients were divided into two groups based on the expression levels of two markers. Specifically, it was not possible to make a comparison-coupled tumor/healthy parenchyma for the whole series, and the normalization of cancers data was obtained by dividing the expression value of each tumor for the median expression values of selected normal breast (K / median N= K / 5,27). Consequently, expression levels of miR-296 were divided into "high" or "low", taking into account the median of the values derived from the normalization (range 0.00018 to 38.01; median 0.10).
Survival curves were generated using the Kaplan-Meier method and compared using the Log Rank test.
Differences in tumor volume between control pre-miR and pre-miR-296-5p injected animals were evaluated using the Mann Whitney U test.
All statistical analyzes were performed using the software Graph Pad Prism version 5 (Graph Pad Software, Inc., La Jolla, California, USA).
A $p \leq 0.05$ was considered statistically significant.

4. RESULTS

4.1 Molecular analysis

To characterize miR-296 behavior in breast cancer, we aimed to investigate the expression levels of this miRNA in a large series of primary breast carcinomas, distant metastases, and normal mammary parenchyma.
We also looked for identify any correlations between its expression values and clinico-pathological parameters, helpful for prognostic and/or predictive determinations.

4.1.1 miR-296 levels and breast cancers

Overall, miR-296 levels were reduced in neoplastic specimens compared to the normal counterpart.
MiR-296 expression was significantly lower in infiltrating breast cancers (mean=3,78; range: 0,0009 to 200,56) than in non-neoplastic mammary parenchyma (mean=19,82, range: 0,02 to 455,93) (p= 0,0044 Student's t test) (Figure 5A).
When patients with distant metastatic lesions were analyzed, miR-296 expression was decreased both in primary tumor (mean=0,24; range: 0,01 to 1,07) and in metastatic lesions (mean=0,96; range 0,01 to 7,52) compared to matched normal glands (p= < 0.0001 Kruskal-Wallis test) (Figure 5B).

4.1.2 miR-296 levels and breast cancers clinico-pathological parameters

Looking at clinico-pathological parameters, as grade, pTNM stage, hormone receptors status or Her-2 expression, no significant correlation could be evidenced with miR-296 levels.
Despite patients' overall survival or disease-free survival were not influenced by miR-296 levels, when we analyzed the group of patients with distant metastases (M1, n=45 cases) we could document a significant correlation between miR-296 levels and the time elapsed for the development of distant metastases.
In fact, lower levels of miR-296 were associated with earlier onset of metastases (p= 0,04 Log rank test) (Figure 6).

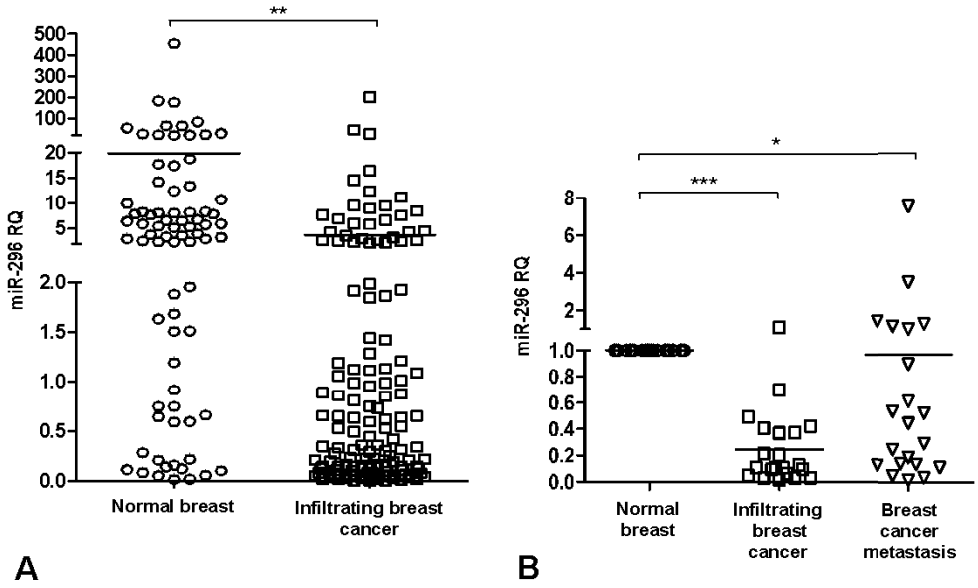

Figure 5. A. Loss of miR-296 expression in infiltrating breast cancers matched with normal breast analyzed by Real Time PCR (RQ relative quantities) (** p< 0,0044 Unpaired t test). Bars represent mean RQ values.
B. MiR-296 levels in primary infiltrating breast cancers and in correspondent distant metastases are reduced compared to normal mammary parenchyma analyzed by Real Time PCR (RQ relative quantities) (*** p< 0,001 and * p < 0,05, Dunn's Multiple Comparison test). Bars represent mean RQ values.

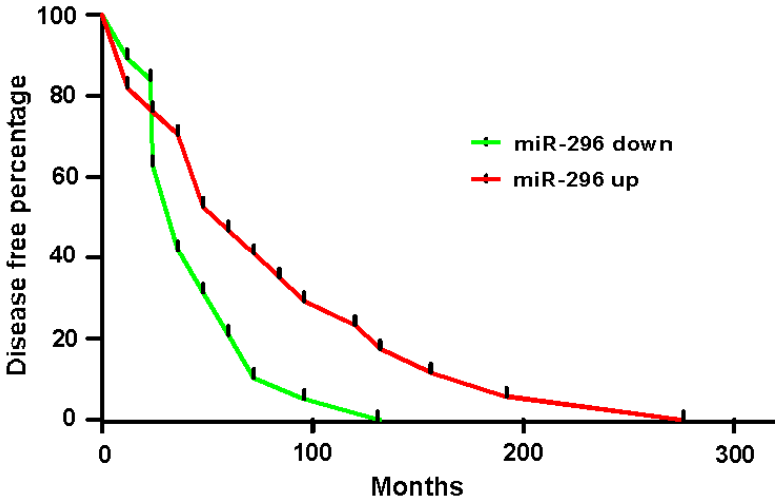

Figure 6. In patients with metastatic (M1) infiltrating breast cancer low miR-296 levels are associated with early development of distant metastases (p= 0,04 log rank test).

4.2 Immunohistochemical analysis

Immunohistochemical stainings on TMA blocks were performed to phenotypically characterize selected infiltrating breast cancers according updated guidelines [245-247] and to assess Scrib protein expression in primary and metastatic lesions.
We evaluated different breast cancer components, ranging from in situ and infiltrating ones to lymph node and distant metastases.
We compared miR-296 expression levels and Scrib expression pattern searching for correlations.
Scrib protein expression intensity and localization were also analyzed in order to identify associations with clinico-pathological breast cancer parameters.

4.2.1 Tumors phenotype

Out of 131 infiltrating breast cancers specimens, 128 resulted adequate for estrogen receptor (ER) evaluation; 113 (86,3%) cases were positive, and 15 (11,4%) negative.
Progesterone receptor showed positive staining in 95 (72,5%) patients out of 131 and a negative one in 32 (24,5%).
Her-2 immunostaining was scored as 0 in 43 (32,8%) specimens out of 131, 1+ in 27 (20,6%), 2+ in 20 (15,3%), and 3+ in 33 (25,2%), respectively.
Ki-67 percentage was comprised between 2 and 70, with an average of 16%.

4.2.2 Scrib expression and breast cancers

Globally, Scrib was over-expressed in breast cancers compared to normal glands.
In situ and infiltrating carcinomas, and lymph node metastases displayed a greater number of positive cases respect to normal parenchyma ($p<0,02$ Fisher's Exact test) (figure 7).
Intensity of Scrib expression was also stronger in primary breast cancers and nodal metastases than in controls ($p<0,0003$ Mann Whitney U test) (Figure 8).
In regards to Scrib sub-cellular localization, in tumor glands prevailed the dual distribution of membrane and cytoplasm of the protein ($p <0,004$ Chi square test and $p=0,004$ Mann Whitney U test) (Figure 9).
Scrib protein mislocalization showed an increasing trend from in situ carcinoma to infiltrating one ($p= 0,09$ Chi square test) (Figure 9).
Similar results were confirmed from the analysis of Scrib expression in a subset of distant breast cancer metastases, irrespective of site (data not shown).
Scrib immunohistochemical expression pattern in primary breast cancers (in situ and infiltrating) and in nodal and liver metastases is depicted in Figure 10.
Summary of Scrib intensity and cellular localization evaluation in breast cancers, nodal metastasis and normal parenchyma is reported in Table 2 and Table 3.

4.2.3 Scrib expression and miR-296

We could not document any statistically significant correlation between miR-296 and Scrib in infiltrating breast cancers, being the number of Scrib positive and negative cases equally distributed within up- and down- regulated miR-296 levels.

4.2.4 Scrib expression and breast cancers clinico-pathological parameters

Considering infiltrating breast cancers grade, tumor dimension, presence or absence of nodal involvement and distant metastasis, site of distant spread, hormone receptors and Her-2 status, we did not observe any association with Scrib protein intensity or cellular localization.
Also overall survival (OS) and disease free survival (DFS) of breast cancer affected patients are not influenced by Scrib protein presence and distribution.

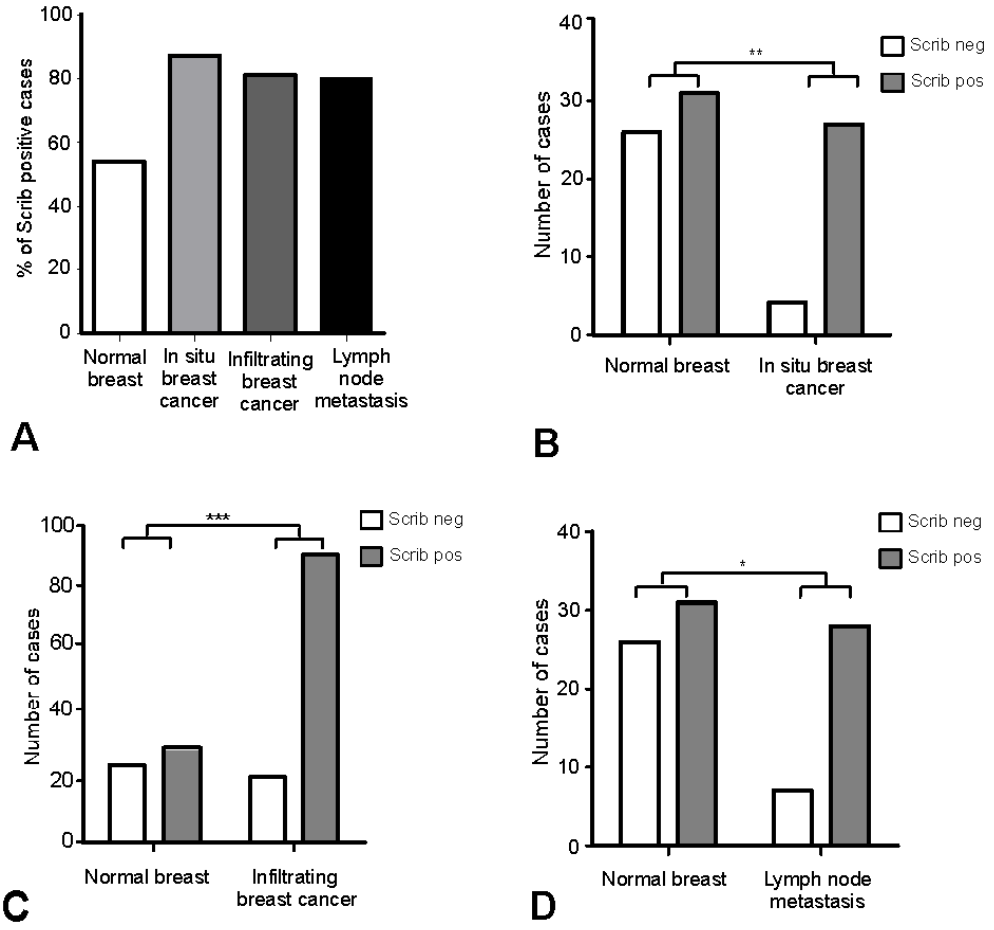

Figure 7. A. In breast carcinomas and in lymph node metastasis Scrib is over-expressed respect to the normal counterpart. B,C,D. In situ breast cancer, infiltrating breast cancer and nodal metastasis display a greater number of positive cases for Scrib compared to the normal mammary parenchyma (** p= 0,0021, *** p = 0,0003, and * p= 0,014 respectively, Fisher's Exact test).

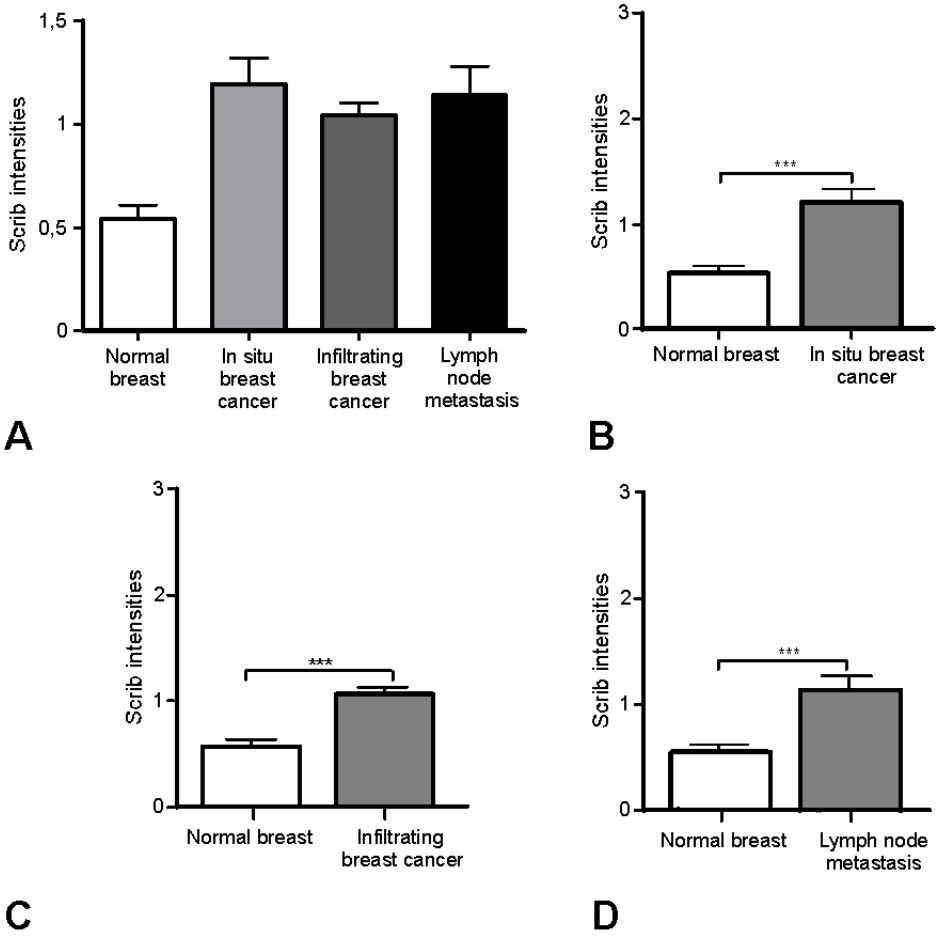

Figure 8. A. Intensity of Scrib expression is more elevated in breast tumors and in lymph node metastasis than in normal breast parenchyma. B,C,D. In situ breast cancer, infiltrating breast cancer and nodal metastasis display stronger Scrib immunoreactivity respect matched controls (*** p <0.0001, *** p <0.0001 and *** p= 0,0002 respectively, Mann Whitney U test).
Scrib intensities were evaluated as follows: negative (0), weak (1), moderate (2), or intense (3).

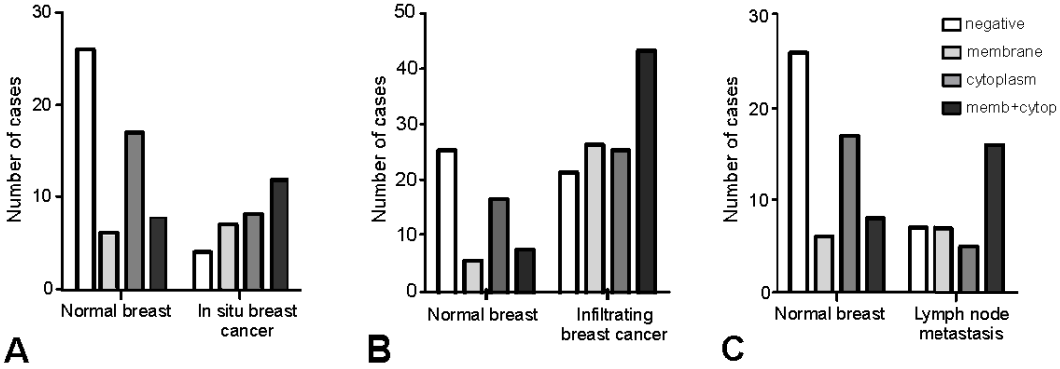

Figure 9. Contemporary cytoplasmic and membranous staining prevails in tumors, also considering separately in situ (A), infiltrating (B) breast cancers and nodal metastases (C), respect to normal mammary lobules.

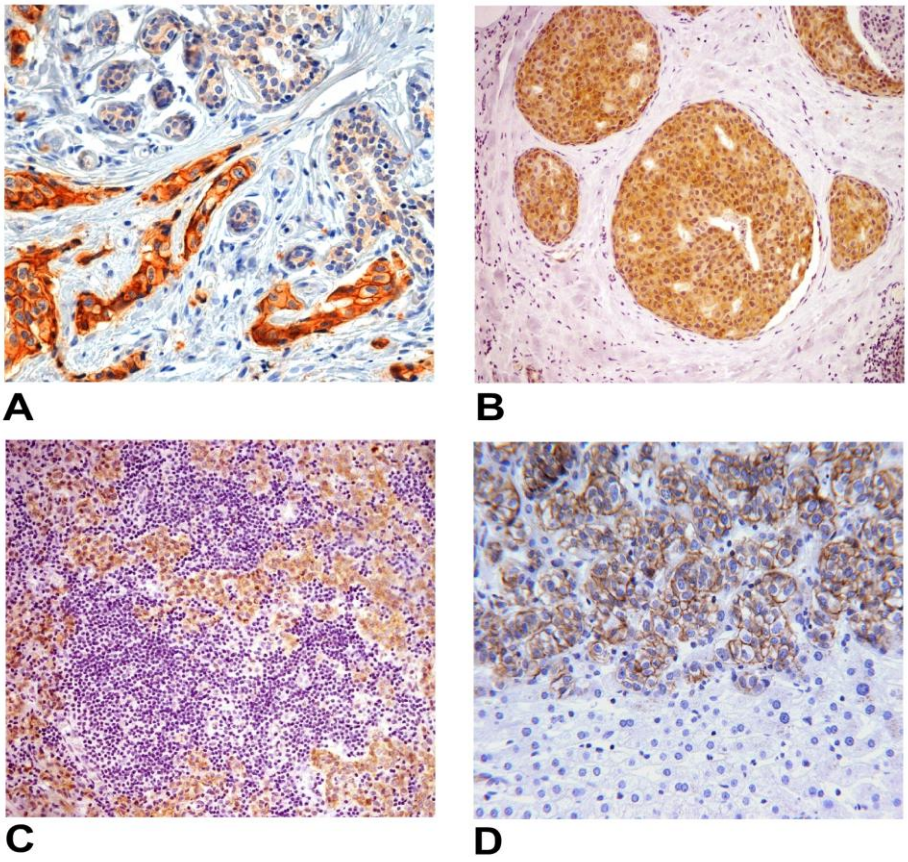

Figure 10. A. Microphotograph showing a stronger Scrib immunostaining in breast cancer glands compared to normal mammary acini. Combination of membranous and cytoplasmic staining is present. Original magnification x200.
B,C,D. Scrib immunohistochemistry for in situ breast cancer (B), nodal metastasis (C) and liver metastasis (D). In situ carcinoma and lymph node metastasis display predominant cytoplasmic and membranous protein localization, liver metastasis a membranous one. Original magnification x200.

	Scrib INTENSITY					
	0	***1***	***2***	***3***	***na****	***Total***
CARCINOMA IN SITU (n°cases)	**4**	**18**	**8**	**1**		***31***
INFILTRATING CARCINOMA (n°cases)	**22**	**72**	**23**	**2**	**12**	***131***
LYMPH NODE METASTASIS (n°cases)	**7**	**18**	**8**	**2**		***35***
NORMAL BREAST (n°cases)	**26**	**31**	**0**	**0**	**20**	***77***

Table 2. Summary of Scrib immunohistochemical intensities in breast cancers (in situ and infiltrating), in lymph node metastasis and in normal mammary parenchyma. Scrib immunoreactivity was evaluated as follows: negative (0), weak (1), moderate (2), or intense (3).

*na= not assessed

	Scrib LOCALIZATION					
	neg	***memb***	***cytop***	***m+c***	***na****	***Total***
CARCINOMA IN SITU (n°cases)	**4**	**7**	**8**	**12**		***31***
INFILTRATING CARCINOMA (n°cases)	**22**	**27**	**26**	**44**	**12**	***131***
LYMPH NODE METASTASIS (n°cases)	**7**	**7**	**5**	**16**		***35***
NORMAL BREAST (n°cases)	**26**	**6**	**17**	**8**	**20**	***77***

Table 3. Summary of Scrib cellular localization in breast cancers (in situ and infiltrating), in lymph node metastasis and in normal mammary parenchyma. Scrib immunostaining was evaluated as follows: negative (neg), membranous (memb), cytoplasmic (cytop), mambranous and cytoplasmic (m+c).

*na= not assessed

4.3 miR-296 levels restoration analysis

miRs act as critical regulators in human disease, and a rapid progress from miR discovery to development of miR-based therapy reflects the importance of their function.

Data emerged from previous [202] and current evidences suggested that miR-296 could play roles in breast carcinogenesis.

We therefore attempted a therapeutic approach delivering a precursor miR-296 molecule into breast cancer tumors in vivo.

Direct injection of pre-miR-296 into tumoral masses of MDA-MB-231 xenograft model significantly hampered breast cancer growth compared with control-treated tumors (Figure 11).

Importantly, administration of a double dose of pre-miR-296 allowed a significant decrease in tumor development, mainly within 8 to 10 days after first delivery ($p<0.05$ Mann Whitney U test) (Figure 11 A).

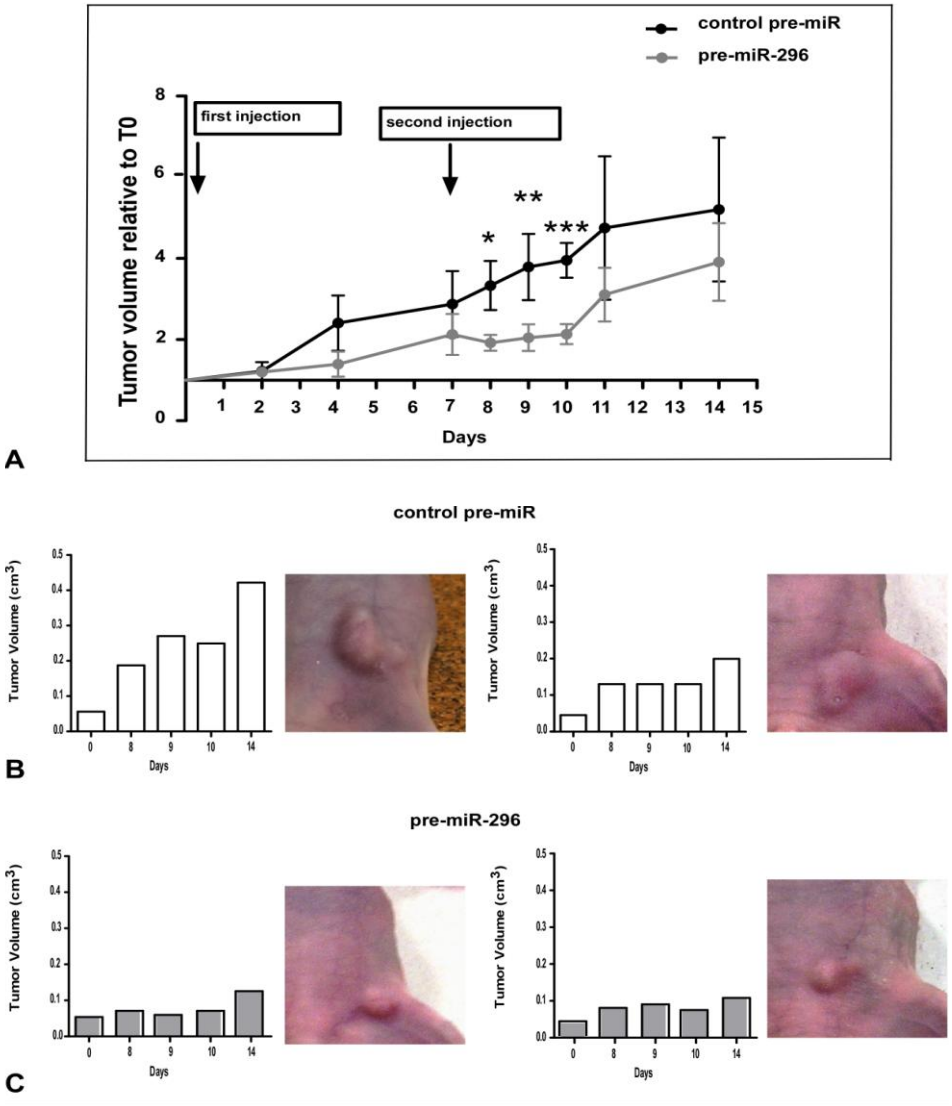

Figure 11. A. Injection of pre-miR-296 into tumoral masses of MDA-MB-231 xenograft model reduced tumor growth, compared with controls, mainly within 8 to 10 days after first delivery (T0) (* p= 0,009, ** p= 0,019, *** p= 0,014 Mann Whitney U test). B,C. Graphs show daily tumor volume measurements in control pre-miR (B) or pre-miR-296 (C) injected mice, from the day of first dose administration until 14 day. Photographs illustrate differences in tumor masses at 14 day in treated or untreated animals.

5. CONCLUSIONS

In this study we demonstrated a significant reduction in expression levels of miR-296 in a large series of primary breast lesions (in situ and infiltrating carcinomas), as well as in metastatic lesions (nodal and distant metastases) compared to normal breast parenchyma. These observations agree with those reported in the literature regarding the reduction of miR-296 in different types of cancer (parathyroid glands, liver, prostate and bladder) compared to the normal counterpart [209,212,248].
However, the role of miR-296 in tumors still remains controversial, with other studies reporting increased levels of this miRNA in human cancers [208].
In breast tumors the available data are not numerous, and recently it has been observed that in cancers with a “triple negative” phenotype (negative for estrogen receptor, progesterone receptor and Her-2) the expression levels of this miR-296 do not vary in comparison to the normal breast tissue [215].
We previously identified in a large series of human cancer cell lines and carcinoma specimens miR-296 as a comprehensive regulator of cell tumorigenicity, migration, and invasion [202], by inhibition of the expression of one of its targets, Scrib, a cytoplasmic protein that participates in multiprotein complexes to maintain the three-dimensional organization of tissues [221-223] and to regulate directional motility [225-227].
Increased levels of Scrib protein, related to a reduction of miR-296, have been reported in a wide range of human tumors, including breast [202,237].
Respect to normal mammary parenchyma, breast cancers also showed changes in Scrib cellular localization, from sites of cell–cell contact to a diffuse cytoplasmic or a combination of membranous and cytoplasmic staining [237,240].
In our study, we observed a significant increase of Scrib positive cases in primary and metastatic breast carcinomas, compared to healthy controls using immunohistochemistry, with a greater expression intensity and a tendency toward a cytoplasmic and membranous localization, but without detecting any correlation with levels of miR-296 or clinico-pathological parameters.
Our data confirm the role of miR-296 in control of cell tumorigenic potential and suggest its participation in cell migration. Indeed, infiltrating breast cancers evidenced a significant reduction of miR-296 levels compared to normal parenchyma.
Identification of miRNAs deregulated during malignant cell transformation or tumor progression can have important implications for cancer therapy and our findings in xenograft assays are promising: injection of pre-miR-296 in nude mice with growing human breast cancer cells resulted effective in slowing tumor growth.
Furthermore, our observation in the group of patients with distant metastases, showing a significant correlation between reduced miR-296 levels and the earlier onset of distant metastases, further suggest an important role of miR-296 in regulation of cell motility, invasiveness and migration.
Despite absence of significant association between miR-296 levels and those of its target Scrib we can confirm our previous results: globally miR-296 and Scrib expression are inversely correlated in normal mammary glands and in breast cancers.
Molecular characterization of diseases is essential to perform personalized medicine and to identify new potential targets for tailored drugs. Our findings suggest that forced enhancement of miR-296 levels could represent a novel therapeutic approach in advanced breast cancer, with the goal of reducing cancer growth and dissemination.

6. REFERENCES

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